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RESEARCH PAPER

Early cytokinin response proteins and phosphoproteins of *Arabidopsis thaliana* identified by proteome and phosphoproteome profiling

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Abstract

Cytokinins are plant hormones involved in regulation of diverse developmental and physiological processes in plants whose molecular mechanisms of action are being intensely researched. However, most rapid responses to cytokinin signals at the proteomic and phosphoproteomic levels are unknown. Early cytokinin responses were investigated through proteome-wide expression profiling based on image and mass spectrometric analysis of two-dimensionally separated proteins and phosphoproteins. The effects of 15 min treatments of 7-day-old *Arabidopsis thaliana* seedlings with four main cytokinins representing hydroxyisopentenyl, isopentenyl, aromatic, and urea-derived type cytokinins were compared to help elucidate their common and specific function(s) in regulating plant development. In proteome and phosphoproteome maps, significant differences were reproducibly observed for 53 and 31 protein spots, respectively. In these spots, 96 proteins were identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), providing a snapshot of early links in cytokinin-regulated signalling circuits and cellular processes, including light signalling and photosynthesis, nitrogen metabolism, the CLAVATA pathway, and protein and gene expression regulation, in accordance with previously described cytokinin functions. Furthermore, they indicate novel links between temperature and cytokinin signalling, and an involvement of calcium ions in cytokinin signalling. Most of the differentially regulated proteins and phosphoproteins are located in chloroplasts, suggesting an as yet uncharacterized direct signalling chain responsible for cytokinin action in chloroplasts. Finally, first insights into the degree of specificity of cytokinin receptors on phosphoproteomic effects were obtained from analyses of cytokinin action in a set of cytokinin receptor double mutants.

Key words: *Arabidopsis thaliana*, cytokinin, phosphoproteome, proteome.

Introduction

Cytokinins were first identified by their ability to promote division in cultured plant cells (Miller *et al.*, 1955). They have since been shown to play roles in diverse aspects of plant growth and development including cell division, shoot initiation, apical meristem function, and vascular formation (Mok and Mok, 2001).

Naturally occurring cytokinins are adenine derivatives substituted at the N^6 position with an isoprenoid or aromatic side chain. Isoprenoid cytokinins are the most abundant cytokinins, while aromatic cytokinins, including N^6-benzyladenine (BA), are minor components of the cytokinin pool (Strnad, 1997). Isoprenoid cytokinins are either of the isopentenyl (iP) type, with an isopentenyl N^6 side chain, or of the zeatin (Z) type, with a hydroxylated isopentenyl N^6 side chain in either *trans* (*t*-Z) or *cis* (*c*-Z) configuration. Reduction of the double bond in the side

chain results in dihydrozeatin (DZ) (Brzobohatý *et al.*, 1994; Mok and Mok, 2001). In addition, structurally unrelated (synthetic) phenylurea-type cytokinins, for example thidiazuron (TDZ), show high activity in most cytokinin bioassays (Mok and Mok, 2001).

The diversity of compounds exerting cytokinin activity might underlie the molecular fine tuning of their numerous functions. Indeed, differences in biological activities of specific cytokinins have long been recognized, for instance in growth and morphogenic responses (e.g. Mok *et al.*, 1978; Sujatha and Reddy, 1998; Lexa *et al.*, 2003), but molecular mechanisms underlying between-cytokinin differences in activity are just emerging.

Between-cytokinin differences in activity can be at least partially explained by differences in the receptors that perceive them and trigger biological responses. Cytokinin perception and signalling apparently evolved from bacterial two-component phosphorelays (Ferreira and Kieber, 2005). Binding of cytokinins to the *Arabidopsis* sensor hybrid histidine kinases AHK2, AHK3, and AHK4/CRE1/WOL1 initiates a phosphorelay in which *Arabidopsis* histidine-containing phosphotransfer proteins (AHPs) are phosphorylated then translocated into the nucleus, where they transfer the phosphate to *Arabidopsis* type-B response regulators (ARRs) (Kakimoto, 2003; Rashotte *et al.*, 2003; Kiba *et al.*, 2005; Choi and Hwang, 2007). The latter play roles in mediating transcriptional responses to cytokinin, including rapid induction of another class of response regulators, type-A ARRs (Rashotte *et al.*, 2003), which act as negative regulators of the primary signal transduction pathway (Argueso *et al.*, 2009). The first evidence for differential ligand specificity of cytokinin receptors has been obtained from their characterization in bacterial expression systems (Spíchal *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004; Romanov *et al.*, 2006).

Global genome expression profiling of cytokinin action in *Arabidopsis* has yielded a genome-wide view of changes in abundance of cytokinin-responsive transcripts that might be relevant for the many biological processes governed by cytokinins (Hoth *et al.*, 2003; Rashotte *et al.*, 2003; Brenner *et al.*, 2005). However, since changes in transcript abundance are not necessarily linearly related to changes in levels and/or activities of corresponding proteins, proteome profiling can provide valuable complementary information regarding molecular mechanisms linking cytokinin signals and their diverse effects in plants. In addition to protein abundance, post-translational modifications (PTMs) of proteins are crucial determinants of protein activity and subcellular location. Phosphorylation is a key PTM; at least 5% of the *Arabidopsis thaliana* genome is involved in regulating protein phosphorylation (Laugesen *et al.*, 2004), indicating that nearly all aspects of cell function may involve reversible phosphorylation.

A set of proteins involved in cytokinin-induced photomorphogenesis has been identified by proteomic analysis (Lochmanová *et al.*, 2008). In addition, rapid alterations of the phosphoproteome following cytokinin treatment have been examined in the moss *Physcomitrella patens* (Heintz *et al.*, 2006), although comprehensive interpretation of the data was hindered by gaps in knowledge of its genome sequence. Nevertheless, our understanding of early cytokinin-responsive proteins and protein PTMs is still rudimentary. Hence, further analysis of proteome and phosphoproteome alterations caused by cytokinins before proteins encoded by the immediate cytokinin response genes (Brenner *et al.*, 2006) accumulate significantly is needed to elucidate aspects of cytokinin signalling and action networks that cannot be deduced solely from transcriptome profiling. Therefore, proteomic analysis was applied to identify early cytokinin response proteins and phosphoproteins in *Arabidopsis* seedlings treated with four main cytokinins—*t*-zeatin (*t*-Z), isopentenyladenine (i-P), 6-benzylaminopurine (BA), and thidiazuron (TDZ). Detection of proteins involved in processes known to be regulated by cytokinins validated the experimental approach, and unexpected cytokinin targets were identified. Contributions of specific cytokinin receptors to the phosphoproteome alterations were assessed by examining effects of the cytokinins in *ahk2ahk3*, *ahk2cre1*, and *ahk3cre1* mutants.

Materials and methods

Plant material, growth conditions, and cytokinin treatment

Seeds of *A. thaliana* ecotype Columbia (Col-0), and *ahk2ahk3*, *ahk2cre1*, and *ahk3cre1* double mutants (provided by Professor Thomas Schmülling, Free University of Berlin) were surface-sterilized and sown on Uhelon 120T (Silk & Progress, Czech Republic) mesh placed on 1% (w/v) agar containing Murashige and Skoog (MS) medium (pH 5.7) supplemented with 5×10^{-4}% (v/v) dimethylsulphoxide (DMSO), stratified at 4 °C for 3 d, and cultivated at 21 °C/19 °C day/night temperatures, with a 16 h photoperiod (90 μmol m^{-2} s^{-1} light intensity) for 7 d. On the seventh day (after the first 2 h of the day period), the Uhelon mesh with the seedlings was transferred onto liquid MS medium supplemented with (i) 5×10^{-4}% (v/v) DMSO (mock buffer); (ii) 5 μM individual cytokinins (BA, TDZ, iP, and *t*-Z; Duchefa) in DMSO (final concentration, as for the mock); (iii) 30 μM D600 and 60 μM $LaCl_3$ (Sigma); or (iv) 30 μM D600, 60 μM $LaCl_3$ (Sigma), and 5 μM *t*-Z, and incubated for 15 min. The concentrations of the calcium signalling inhibitors (D600 and $LaCl_3$) followed Saunders and Hepler (1983) who observed disruption of cytokinin-induced bud formation in the moss *Funaria* in response to them. Seedlings were rapidly harvested, dried, then frozen and ground in liquid nitrogen.

Protein extraction

Total protein was extracted from frozen seedlings (250–300 mg) by acetone/trichloroacetic acid (TCA) extraction (Damerval *et al.*, 1986). Dried protein was solubilized for 2 h at 30 °C in SOL buffer: 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 90 mM dithiothreitol (DTT). Insoluble matter was removed by centrifugation (15 000 *g* for 10 min) and the protein concentration was determined (Bradford, 1976) (Sigma-Aldrich, http://www.sigmaaldrich.com/) after diluting 1 μl of the total protein extract in 1 ml of reaction mix to prevent the SOL buffer interfering with the Bradford assay. Solubilized protein was then diluted 1:1 with rehydration solution [SOL supplemented with 1% (v/v) ampholytes pH 3–10, 0.2% (w/v) bromophenol blue] and loaded onto IPG strips (Bio-Rad, http://www.bio-rad.com/).

For phosphoproteome analysis, an isolation procedure was established using a PhosphoProtein Purification Kit (Qiagen,

http://www.qiagen.com/). Briefly, 350–400 mg of seedlings ground in liquid nitrogen were extracted with 4 ml of lysis buffer supplemented with protease inhibitors and benzonase (Qiagen kit). Each sample was then diluted to 25 ml with lysis buffer, applied to an affinity column and processed according to the supplier's manual (Qiagen). Protein concentration was determined by the Bradford assay. Desalted phosphoproteins in TRIS-HCl buffer (pH 7.0) were diluted with rehydration solution:SOL (1:1) and loaded onto IPG strips.

2D gel electrophoresis

Proteins were separated essentially as previously described (Lochmanová *et al.*, 2008). Briefly, portions containing 500 μg of protein or 150 μg of phosphoprotein were applied to 18 cm and 7 cm IPG strips, respectively, with a linear pH gradient (4–7), the strips were rehydrated for 16 h at room temperature in buffer containing the extracts, then the proteins were isoelectrically focused at 22 °C in six steps in a PROTEAN IEF Cell unit (Bio-Rad): 150 V (30 min), 300 V (60 min), 600 V (60 min), 1500 V (180 min), 3500 V (300 min), and 10 000 V to 80 000 Vh for long strips; 150 V (20 min), 300 V (20 min), 600 V (20 min), 1500 V (20 min), 3000 V (20 min), and 4000 V up to 12 000 Vh for short strips. The strips were then treated with buffers containing DTT and iodoacetamide (Sigma-Aldrich) to reduce and alkylate the proteins, which were then separated by 11% polyacrylamide SDS–PAGE with the following settings: 50 V (120 min) followed by 100 V (16 h) for large gels (proteome analysis), and 100 V (10 min) followed by 150 V (50 min) for small gels (phosphoproteome analysis), using a PROTEAN Plus Dodeca Cell, and a Mini-PROTEAN 3 Dodeca Cell (Bio-Rad), respectively.

Protein staining and image analysis

Gels were stained with colloidal Bio-Safe Coomassie G-250 (Bio-Rad) and scanned with a Bio-Rad GS-800 Calibrated Densitometer (400 dpi and 700 dpi for large and small gels, respectively). Acquired images were analysed using Decodon Delta 2D software (http://www.decodon.com). Three, six, three, and four biological replicates were used in the 2-DE total proteome comparisons of the wild type, phosphoproteome comparisons of the wild type, phosphoproteome comparisons of the *ahk* double mutant, and phosphoproteome comparisons of wild-type samples in the presence of calcium signalling inhibitors, respectively. Cytokinin responses of proteins corresponding to detected spots were deemed significant if there was a cytokinin/mock, BA/TDZ, BA/iP, or BA/*t*-Z spot volume ratio of ±1.4 or more (for at least one variant), with *t*-test values ⩾95% and similar profiles in (i) ⩾2 biological replicates for total protein comparisons (with three parallel SDS–PAGE analyses for each treatment, i.e. 15 parallel SDS–PAGE analyses for each biological replicate); (ii) ⩾3 biological replicates for phosphoproteome comparisons (with two parallel SDS–PAGE analyses per treatment, i.e. 10 parallel SDS–PAGE analyses for each biological replicate); (iii) three biological replicates for phosphoproteome comparisons in the *ahk* double mutants (with two parallel SDS–PAGE analyses per treatment, i.e. 8 parallel SDS–PAGE analyses for each biological replicate); or (iv) ⩾2 biological replicates for phosphoproteome comparisons in the wild type in the presence of calcium signalling inhibitors. Only spots with significant and reproducible changes were considered for mass spectroscopic identification. The experimental design is outlined schematically in Supplementary Fig. S1 available at *JXB* onluine.

Protein identification

Proteins were identified as previously described (Hradilová *et al.*, 2010) with minor modifications. Briefly, selected protein spots were digested with trypsin. The dried tryptic peptides were each dissolved in 10 μl of 0.1% trifluoroacetic acid and purified using ZipTip C18 tips. The eluate was mixed with 1 vol. of 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (CHCA) in 50% (v/v) acetonitrile and 0.1% trifluoroacetic acid for spotting onto sample plates, and dried for matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis. To demonstrate phosphorylation of selected peptides, phosphopeptides were first enriched from tryptic peptides dissolved in 10% acetonitrile and 0.1% acetic acid using IMAC tips (Millipore) containing iron ions. After loading, the tips were washed with 10% acetonitrile and 0.1% acetic acid, then rinsed with water. Phosphopeptides were eluted by 0.3 N ammonium hydroxide and measured using 15 mg ml^{-1} 2,5-dihydroxybenzoic acid in 50% (v/v) acetonitrile and 6% phosphoric acid solution as a matrix.

Alkaline phosphatase treatment was used to confirm the phosphorylation of the phosphopeptides according to Larsen *et al.* (2001). Briefly, IMAC-purified phosphopeptides were incubated with 0.05 U μl^{-1} alkaline phosphatase in 50 mM NH_4HCO_3, pH 7.8 at 37 °C for 30 min then acidified with 2.5 μl of 5% trifluoroacetic acid. Phosphopeptides were identified by single or multiple 80 Da (HPO_3) losses in MALDI-TOF-MS following alkaline phosphatase treatment, for mono- and multiphosphorylated peptides, respectively.

MALDI-TOF/TOF measurements were performed with an Applied Biosystems 4700 Proteomic Analyzer (Applied Biosystems, http://www.appliedbiosystems.com/) equipped with an Nd:YAG laser (355 nm) operated with 3–7 ns pulses and 200 Hz firing rate in positive reflectron mode for both MS and MS/MS analyses. The accelerating voltage in the ion source for MS and MS/MS analyses was set at 20 kV and 8 kV, respectively. Acquired sequences were searched against the NCBInr sequence database (version 09/2009) using Mascot (http://www.matrixscience.com/), and peaks generated from the acquired mass spectra by the Peak-to-Mascot function incorporated in the software. In the MS analyses, peaks in the 900–4000 m/z range with signal to noise (S/N) ratios >4 were sought. In the MS/MS analyses, peaks with S/N ratios >4 in the m/z range from 68 m/z up to 20 m/z units lower than their precursors' m/z values were used. The resulting peak lists contained information from both MS and MS/MS runs concerning fragmentation patterns of selected precursors. Parameters for both MS and MS/MS data searches in Mascot were: taxonomy, *Arabidopsis thaliana*; enzyme, trypsin; allowed missed cleavages, 1 [except for the peptide VGKDSKDKELKEAFK of endoplasmin homologue (SHD), where allowed missed cleavages were set to 4]; fixed modification, carbamidomethyl (C); variable modifications, none or Phospho (ST) and Phospho (Y) (for searching phosphopeptides); peptide tolerance, 0.5 Da; MS/MS tolerance, 0.5 Da; peptide charge, (+1); instrument, MALDI-TOF/TOF. Protein matches in MS/MS identification were considered valid if there was at least one peptide with a Mascot score corresponding to identity or extensive homology (P <0.05). Protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits by Mascot. Similar parameters were set for peptide mass fingerprint analysis—only protein matches with Mascot scores indicating extensive homology were accepted.

Gene ontology

Gene ontology was evaluated by BiNGO 2.3 in Cytoscape 2.6.2, with data from the NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (http://www.arabidopsis.org) databases.

Results

Identification of early cytokinin response proteins

To identify early cytokinin response proteins, 7-day-old *Arabidopsis* seedlings were treated (separately) with four main cytokinins (BA, iP, TDZ, and *t*-Z) at a concentration

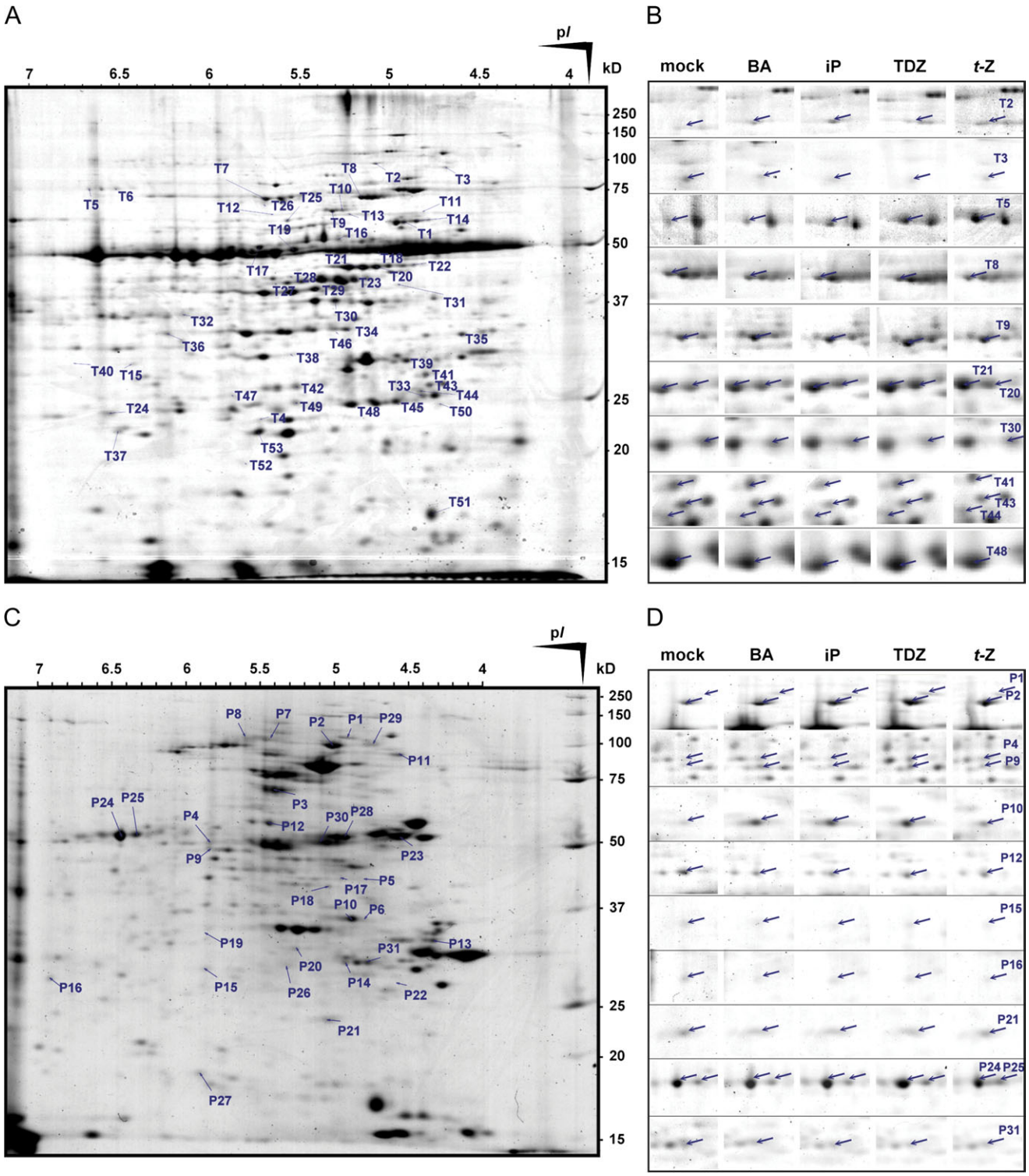

Fig. 1. Effects of cytokinin treatment on the proteome and phosphoproteome of *Arabidopsis* seedlings. (A) Average two-dimensional gel electrophoresis proteome map of 7-day-old *Arabidopsis* seedlings treated with cytokinin/mock buffer for 15 min. Differentially regulated protein spots are indicated. See Table 1, and Supplementary Table S1 at *JXB* online, for detailed information on the corresponding identified proteins. Proteins (500 μg) were separated in the first and second dimensions by IPG (18 cm strips, pH 4–7) followed by 11% SDS–PAGE then visualized by Bio-Safe Coomassie G250 staining. Isoelectric points (pI) and migrating positions of molecular mass (kDa) markers are marked. (B) Examples of spots corresponding to the differentially regulated proteins in *Arabidopsis* seedlings treated with 5 μM cytokinin (BA, iP, TDZ, or *t*-Z) or mock buffer for 15 min. For details see Materials and methods. (C) Average 2-DE phosphoproteome map of 7-day-old *Arabidopsis* seedlings treated with cytokinin/mock buffer for 15 min. Differentially regulated protein spots are indicated. See Table 2, and Supplementary Table S2, for detailed information on the corresponding identified proteins. Phosphoprotein fractions were obtained using a PhosphoProtein Purification Kit. Phosphoproteins (150 μg) were separated in the first

of 5 μM for 15 min. Total proteins were then extracted and subjected to 2-DE (Fig. 1A, B). Image analysis of the resulting proteome maps revealed >850 reproducibly resolved spots in gels over pI and molecular mass ranges of 4–7 and 10–120 kDa, respectively, then proteome patterns of seedlings treated with the individual cytokinins were compared separately with the proteome patterns of seedlings treated with mock buffer. Significant differences (P <0.05) in all biological replicates were found for 160 resolved spots, but only 53 spots were reproducibly significant in two or more independent experiments and were then subjected to protein identification. Altogether, 67 proteins were identified in the 53 spots, including 10 protein mixtures and a non-dissociated heterodimer consisting of small and large Rubisco subunits (T13), by MALDI-TOF/TOF MS analysis followed by Mascot database searches of the full NCBI Arabidopsis protein database (Table 1; Supplementary Table S1 at *JXB* online). The ratio of numbers of up-regulated to down-regulated proteins was ~1:2. Identified protein spots are marked in protein maps shown in Fig. 1A, and corresponding partial amino acid sequences are listed in Supplementary Table S3. Protein identifications and relative fold changes based on mean percentage volumes of each of these spots are presented in Table 1. The apparent strength of effects of the cytokinins on expression of the early cytokinin response proteins decreased in the order BA=TDZ>*t*-Z=iP.

Previously, cytokinin early response transcripts were identified following 15 min treatment of 7-day-old *Arabidopsis* seedlings with 5 μM BA. Here it was confirmed that levels of type-A *ARR* genes (*ARR3* and *ARR5*) increased following BA treatment in the experimental set-up employed using quantitative RT-PCR (P. Souček, unpublished data) as outlined in Souček *et al.* (2007).

Identification of early cytokinin response phosphoproteins

A fraction of phosphoproteins phosphorylated at serine and threonine residues was isolated from seedlings treated with cytokinins using a Quiagen Phosphoprotein enrichment kit with an optimized procedure, as outlined above. In addition, cytokinin receptor double mutants (*ahk2ahk3*, *ahk2cre1*, and *ahk3cre1*) treated with 5 μM *t*-Z for 15 min were analysed to assess how much the individual cytokinin receptors contribute to phosphoproteome regulation. Phosphoprotein fractions were subjected to 2-DE, and image analysis was used to reveal phosphoproteins differentially regulated by cytokinins (Fig. 1C, D), essentially as described above for early cytokinin response proteins. Of 450 reproducibly resolved spots in phosphoproteome maps from wild-type samples, significant differences (P <0.05) in all 10 independent experiments (including the four pilot experiments using only one cytokinin each) were found for 90 resolved spots (for a schematic representation of the experimental design see Supplementary Fig. S1 at *JXB* online). Reproducible significant changes in at least three biological replicates were found for 31 spots. Subsequently, >90% of them were reproducibly resolved in phosphoproteome maps displaying phosphoproteins from each of the three cytokinin receptor double mutants (Table 3).

In total, 29 proteins were identified in these spots, including two protein mixtures, by MALDI-TOF/TOF MS followed by Mascot database searches of the full NCBI protein database (Table 2; Supplementary Table S2 at *JXB* online). Phosphorylation has been previously reported for 22 of these proteins (Table 2; PhosPhAt 3.0, http://phosphat.mpimp-golm.mpg.de). Here, phosphorylation was confirmed for 60S acidic ribosomal protein P0-2 (P22) and endoplasmin homologue (SHD; P2) by comparing MS spectra of their IMAC-purified peptides VEEKEESDEEDYGGDFGLFDEE and VGKDSKDKELKEAFK, respectively, before and after alkaline phosphatase treatment (Supplementary Fig. S2). Serine was previously shown to be a phosphorylation site on the peptide of 60S acidic ribosomal protein P0-2 (Laugesen *et al.*, 2006), but phosphorylation of endoplasmin homologue (SHD) has not been previously reported. In addition, 29 of the 31 spots were stained by the phosphoprotein-specific stain Phostag™ (Supplementary Fig. S3). The ratio of numbers of up-regulated to down-regulated phosphoproteins was ~1:1. Alterations in levels of individual phosphoproteins may result from phosphorylation/dephosphorylation events and/or modulation of turnover rates of the phosphoproteins. Identified protein spots are marked in protein maps shown in Fig. 1C, and examples of spots containing phosphoproteins differentially regulated by the individual cytokinins in Fig. 1D. The corresponding partial amino acid sequences are listed in Supplementary Table S4. Protein identifications and relative fold changes based on mean percentage volumes of these spots are presented in Tables 2 and 3 for phosphoproteins of the wild type and cytokinin receptor double mutants, respectively. Apparent strength of effects of the cytokinins on expression of the early cytokinin response phosphoproteins decreased in the order BA>TDZ>*t*-Z=iP. Responses specific for a single receptor were found in 15 spots, while seven spots were regulated by two individual receptors (Table 3). The remaining spots were either non-significantly or inconsistently (down-, up-, and non-regulated in the individual biological replicas) regulated. Interestingly, regulation was apparent in the mutants for four spots (P19, P24, P25, and P26) that remained below cut-off limits in wild-type seedlings. The opposite regulation was found for spots P13, P26 (*ahk2ahk3*

and second dimensions by IPG (7 cm strips, pH 4–7) followed by 11% SDS–PAGE then visualized by Bio-Safe Coomassie G250 staining. Isoelectric points (pI) and relative migrating positions of molecular mass (kDa) markers are marked. (D) Examples of spots corresponding to the differentially regulated phosphoproteins in *Arabidopsis* seedlings treated with 5 μM cytokinin (BA, iP, TDZ, or *t*-Z) or mock buffer for 15 min. For details see Materials and methods.

Table 1. *Early cytokinin response proteins of* Arabidopsis

Spot/protein no.	AGI code	Protein name	MALDI-MS (protein score/%cov/pep#)	Relative fold change			
				BA	iP	TDZ	*t*-Z
T1	At2g28000	Rubisco large subunit-binding protein subunit α, chloroplastic	666/32/12	–1.5±0.23	–1.5±0.23	–1.4±0.21	–1.4±0.21
T2	At4g24190	Endoplasmin homologue (SHD)	27/1/1	1.5±0.10	1.8±0.20	1.6±0.32	1.4±0.28
T3	At5g56000	Heat shock protein 81-4	153/8/5	–2.0±0.20	–1.5±0.23	–2.0±0.30	–1.3±0.20
T4	At1g19570	Glutathione *S*-transferase DHAR1, mitochondrial	35/17/2	–1.4±0.21	–1.3±0.20	–1.3±0.20	–1.5±0.23
T5	At5g17920	Cobalamin-independent methionine synthase	49/6/3 PMF: 127/23/11	1.4±0.14	1.4±0.26	1.6±0.33	1.7±0.30
T6	At5g17920	Cobalamin-independent methionine synthase	55/7/3 PMF: 104/21/9	1.3±0.26	1.4±0.28	1.5±0.30	1.4±0.21
T7	At3g60750	Putative transketolase	236/17/4	–1.5±0.10	–1.5±0.30	–1.6±0.30	–1.4±0.20
T8	At5g02500	Heat shock cognate 70 kDa protein 1	104/10/3	–1.3±0.20	–1.4±0.21	–1.5±0.23	–1.3±0.20
T9	At2g30950	Cell division protease FtsH homologue 2, chloroplastic	98/8/3	1.5±0.30	1.4±0.28	1.3±0.26	1.4±0.21
T10	At2g30950	Cell division protease FtsH homologue 2, chloroplastic	101/9/3	1.4±0.10	1.7±0.12	1.5±0.30	1.8±0.10
T11	At5g60640	Protein disulphide isomerase-like protein	96/25/8	–2.5±0.38	–2.0±0.30	–1.6±0.24	–2.0±0.30
T12	AtCg00120	ATP synthase subunit α, chloroplastic	114/26/9	1.3±0.26	1.4±0.28	1.4±0.21	1.4±0.21
T13	At5g38420	Rubisco small chain 2β, chloroplastic	45/8/1	–1.4±0.21	–1.3±0.20	–1.3±0.20	–1.4±0.21
	AtCg00490	Rubisco large chain	PMF: 159/34/14				
T14	At1g21750	Probable protein disulphide-isomerase 1	41/6/2	–1.7±0.23	–1.4±0.21	–1.6±0.30	–1.4±0.15
T15	At1g20020	Ferredoxin-NADP reductase, leaf 2, chloroplastic	383/29/7	1.6±0.32	1.3±0.30	1.0±0.30	1.0±0.32
T16	AtCg00120	ATP synthase subunit α, chloroplastic	238/20/6	1.5±0.30	1.2±0.24	1.2±0.22	1.4±0.28
T17	At5g08690	ATP synthase subunit β-2, mitochondrial	84/12/4	–1.3±0.20	–1.2±0.20	–1.2±0.18	–1.5±0.23
T18	AtCg00490	Rubisco large chain	269/22/7	2.0±0.40	1.5±0.30	2.0±0.40	1.5±0.30
T19	AtCg00480	ATP synthase subunit β, chloroplastic	128/10/4	–1.3±0.24	–1.4±0.30	–1.5±0.23	–1.4±0.21
T20	At2g39730	Rubisco activase, chloroplastic	714/39/10	1.4±0.28	1.5±0.34	1.5±0.30	1.4±0.25
T21	At2g39730	Rubisco activase, chloroplastic	465/17/5	1.4±0.24	1.5±0.30	1.3±0.26	1.6±0.25
T22	At3g54050	Fructose-1,6-bisphosphatase, chloroplastic	100/12/4	–1.3±0.15	–1.2±0.18	–1.5±0.08	–1.5±0.10
T23	At5g35630	Glutamine synthetase, chloroplastic/mitochondrial	PMF: 72/29/5	–1.3±0.20	–1.2±0.18	–1.4±0.21	–1.2±0.18
T24	At4g02520	Glutathione *S*-transferase PM24	230/45/8	1.7±0.20	1.9±0.10	1.5±0.30	1.3±0.26
	At5g61410	Ribulose-5-phosphate-3-epimerase	302/19/3				
T25	At1g09780	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase 1	435/25/9	1.4±0.28	–1.2±0.13	–1.4±0.20	–1.3±0.20
T26	At5g26000	Myrosinase	PMF: 61/13/5	–1.5±0.24	–1.6±0.24	–1.5±0.23	–1.3±0.12
T27	At3g18780	Actin-2	147/23/5	–1.6±0.24	–1.5±0.14	–1.5±0.23	–1.4±0.22
	At1g49240	Actin-8	147/23/5				
	At5g35630	Glutamine synthetase, chloroplastic/mitochondrial	133/15/3				
T28	At4g20360	Elongation factor Tu, chloroplastic	66/7/2	–1.5±0.23	–1.3±0.20	–1.8±0.26	–1.5±0.10
T29	At2g39730	Rubisco activase, chloroplastic	396/22/5	1.4±0.20	1.4±0.15	1.4±0.30	1.4±0.21
T30	At1g32060	Phosphoribulokinase, chloroplastic	287/26/7	–1.6±0.15	–1.4±0.22	–1.5±0.10	–1.5±0.20
T31	At3g12780	Phosphoglycerate kinase	254/17/5	–1.5±0.20	–1.5±0.10	–1.5±0.10	–1.6±0.20
T32	At3g52930	Fructose-bisphosphate aldolase	318/23/6	1.6±0.32	1.7±0.21	2.1±0.42	2.2±0.30
T33	At2g43910	Thiol methyltransferase, putative	64/13/2	–1.8±0.27	–1.6±0.24	–1.1±0.22	–1.2±0.30
T34	At3g09200	60S Acidic ribosomal protein P0-2	59/14/2	–1.4±0.21	–1.7±0.22	–2.1±0.31	–2.0±0.30
T35	At1g30230	Elongation factor 1- δ 1	897/46/8	–1.5±0.20	–1.4±0.23	–1.5±0.15	–1.4±0.21
T36	At2g05990	Enoyl-[acyl-carrier-protein] reductase	206/23/6	1.7±0.15	1.7±0.34	1.6±0.32	1.7±0.02

Table 1. *Continued*

Spot/protein no.	AGI code	Protein name	MALDI-MS (protein score/%cov/pep#)	Relative fold change			
				BA	iP	TDZ	*t*-Z
T37	At3g10920	Superoxide dismutase [Mn], mitochondrial	462/38/7	–1.2±0.19	–1.4±0.08	–1.4±0.21	–1.3±0.20
	At2g47730	Glutathione *S*-transferase 6, chloroplastic	147/35/4				
T38	At3g16420	PBP1	35/6/1	–1.4±0.21	–1.2±0.18	–1.4±0.21	–1.3±0.20
T39	At3g53460	29 kDa ribonucleoprotein, chloroplastic	71/4/1	–1.4±0.22	–1.4±0.21	–1.6±0.24	–1.4±0.21
T40	At4g28520	Cruciferin 3	475/41/10	–2.4±0.36	2.7±0.54	1.1±0.30	–1.3±0.20
T41	At5g38480	14-3-3-like protein GF14 ψ	403/39/6	–1.5±0.23	–1.3±0.20	–1.6±0.25	–1.2±0.18
	At1g22300	14-3-3-like protein GF14 ϵ	326/26/5				
T42	At5g14740	β-Carbonic anhydrase 2	333/35/5	1.6±0.20	1.6±0.10	1.7±0.34	1.9±0.38
T43	At2g37220	Putative ribonucleoprotein, chloroplastic	440/24/6	–1.4±0.21	–1.2±0.11	–1.1±0.17	–1.2±0.18
	At5g50250	Putative 31 kDa ribonucleoprotein, chloroplastic	85/7/2				
T44	At5g10450	14-3-3-like protein GF14 λ	39/9/1	1.9±0.35	1.5±0.30	1.4±0.24	1.4±0.28
T45	At2g34430	Photosystem II type I chlorophyll *a*/*b*-binding protein (LHB1B1)	229/31/3	–1.6±0.24	–1.7±0.26	–1.1±0.20	–1.5±0.23
	At2g34420	Photosystem II type I chlorophyll *a*/*b*-binding protein (LHB1B2)	229/31/3				
T46	At2g21330	Fructose-bisphosphate aldolase (FBA1)	105/12/3	–1.2±0.23	–1.3±0.20	–1.5±0.23	–1.6±0.24
	At4g38970	Fructose-bisphosphate aldolase (FBA2)	76/19/4				
T47	At1g54870	Glucose and ribitol dehydrogenase homologue 1	PMF: 61/31/5	–1.5±0.23	–1.5±0.23	–1.5±0.23	–1.4±0.21
T48	At1g29910, At1g29920	Chlorophyll *a*-*b*-binding protein 165/180, chloroplastic (CAB2/3)	419/35/4	–1.5±0.23	–1.5±0.30	–1.4±0.21	–1.3±0.20
	At1g29910, At1g29930	Chlorophyll *a*-*b*-binding protein 2, chloroplastic (CAB1)					
T49	At3g55440	Triosephosphate isomerase, cytosolic	122/23/3	–1.4±0.22	–1.5±0.23	–2±0.30	–1.4±0.15
T50	At3g14290	Proteasome subunit α type-5-B	121/27/4	–1.5±0.23	–1.7±0.30	–1.2±0.20	–1.5±0.23
	At1g53850	Proteasome subunit α type-5-A	88/21/3				
T51	At3g27830	50S Ribosomal protein L12-1, chloroplastic	313/36/3	–1.6±0.18	–1.1±0.17	–1.3±0.20	–1.3±0.20
	At3g27850	50S Ribosomal protein L12-3, chloroplastic					
T52	At4g38680	Glycine-rich protein 2/cold shock domain protein 2	74/21/2	–1.6±0.10	–1.5±0.23	–1.6±0.24	–1.3±0.20
T53	At1g61520	LHCA3 (PSI type III chlorophyll *a*/*b*-binding protein); chlorophyll binding	207/13/3	–1.3±0.20	–1.4±0.20	–1.3±0.32	–1.7±0.26

Spot no., spot number (as given in Fig. 1A); AGI code, accession number in the TAIR database; Protein name, entry name in the NCBI database; %cov, percentage of protein coverage; pep#, number of peptides; PMF, peptide mass fingerprint; Relative fold change, fold change relative to the mock control (calculated by DECODON DELTA 2D software) ±SE. Full information on the proteins including their classification, peptide sequences and peak list is given in Supplementary Tables S1 and S3 at *JXB* online.

Table 2. *Early cytokinin response phosphoproteins of* Arabidopsis

Spot/protein no.	AGI code	Protein name	PhosPhAt database	MALDI-MS (protein score/%cov/pep#)	Relative fold change			
					BA	iP	TDZ	*t*–Z
P1	At1g22530	Patellin-2 (PATL-2)	+	42/1/3	2.0±0.05	2.1±0.10	1.8±0.08	1.9±0.06
P2	At4g24190	Endoplasmin homologue (SHD)		76/10/7	1.9±0.07	1.4±0.11	1.7±0.09	1.7±0.20
P3	At5g56030	Heat shock protein 81-2	+	227/10/5	2.0±0.15	1.7±0.06	1.6±0.05	2.0±0.15
P4	At5g11170	DEAD-box ATP-dependent RNA helicase 15		33/4/2	1.6±0.18	1.7±0.05	1.8±0.06	1.5±0.15
P5	At5g22650	Histone deacetylase HDT2	+	86/11/3	1.6±0.13	2.0±0.31	2.3±0.28	1.6±0.18
P6	X	X	?	X	2.3±0.15	2.5±0.34	2.5±0.26	2.0±0.21
P7	X	X	?	X	1.7±0.15	2.0±0.32	1.7±0.21	2.5±0.13
P8	X	X	?	X	2.2±0.10	1.7±0.09	1.6±0.05	1.4±0.08
P9	At1g09640	Probable elongation factor 1-γ 1		84/10/3	2.0±0.08	1.6±0.06	1.5±0.07	1.5±0.05
P10	At1g76180	Dehydrin ERD14	+	50/20/2	1.7±0.04	2.1±0.13	2.3±0.15	1.3±0.07
P11	At5g60640	Protein disulphide isomerase-like protein		333/22/9	–1.5±0.05	–1.4±0.07	–1.6±0.09	–1.8±0.09
P12	AtCg00120	ATP synthase subunit α, chloroplastic	+	701/25/9	–1.3±0.06	–1.8±0.11	–1.5±0.10	–1.5±0.06
P13	AtCg00490	Rubisco large chain	+	80/12/5	–1.5±0.09	–1.7±0.15	–1.9±0.12	–2.0±0.23
	At1g67090	Rubisco small chain 1A, chloroplastic	+	78/19/3				
P14	At2g39990	eIF2 (eukaryotic translation initiation factor)		139/17/3	–1.6±0.05	–1.5±0.17	–1.2±0.25	–2.0±0.12
P15	At5g14740	β-Carbonic anhydrase 2	+	383/25/4	–1.5±0.08	–1.5±0.09	–1.4±0.08	–1.4±0.10
P16	At5g43830	GATase-like protein	+	119/9/2	–1.4±0.04	–2.0±0.06	–1.5±0.05	–1.5±0.10
P17	At5g56030	Heat shock protein 81-2	+	574/19/11	–2.4±0.20	–2.5±0.33	–2.6±0.24	1.0±0.30
P18	At5g56030	Heat shock protein 81-2/3/4	+	63/8/4	–1.7±0.08	–1.6±0.06	–2.0±0.30	–1.5±0.05
P19	At3g16420	PBP1	+	70/12/2	–2.5±0.35	–1.8±0.18	–1.8±0.25	–1.3±0.20
P20	At5g42790	Proteasome subunit a type-1-A		94/27/4	–1.9±0.23	–2.0±0.15	–1.5±0.08	–1.5±0.08
P21	At3g51880	HMGB1	+	48/7/2	–1.6±0.10	–1.4±0.12	–1.3±0.07	–1.6±0.14
P22	At3g09200	60S Acidic ribosomal protein P0-2	+	124/18/3	–2.5±0.22	–2.0±0.32	–2.0±0.21	–1.2±0.20
P23	At1g20440	Dehydrin COR47	+	PMF: 106/43/9	1.7±0.08	1.6±0.15	1.3±0.15	1.1±0.18
	At4g26110	NAP1	+	PMF: 61/23/7				
P24	AtCg00490	Rubisco large subunit	+	291/22/7	1.6±0.10	1.6±0.08	1.4±0.18	1.3±0.15
P25	AtCg00490	Rubisco large subunit	+	317/16/7	–1.6±0.07	–1.6±0.05	–1.2±0.40	1.3±0.15
P26	At1g76180	Dehydrin ERD14	+	87/25/3	–1.8±0.05	–1.6±0.08	–1.6±0.05	–1.3±0.16
P27	At1g26630	eIF5A-2 (eukaryotic translation initiation factor)	+	95/28/3	1.7±0.08	1.4±0.14	1.3±0.10	1.5±0.05
P28	At1g20010	Tubulin β-5 chain		223/14/5	1.6±0.11	1.5±0.29	1.8±0.07	1.6±0.07
P29	X	X	?	X	1.3±0.23	1.5±0.07	1.7±0.05	1.7±0.05
P30	At5g44340	Tubulin β-4 chain	+	488/23/11	1.4±0.15	1.4±0.05	1.5±0.04	1.4±0.15
P31	At3g09200	60S Acidic ribosomal protein P0-2	+	309/16/3	–1.3±0.20	–1.4±0.08	–1.5±0.10	–1.6±0.10

Spot no., spot number (as given in Fig. 1C); AGI code, accession number in the TAIR atabase; PhosPhAt, the Arabidopsis Protein Phosphorylation Site Database (Heazlewood *et al.*, 2008); Protein name, entry name in the NCBI database; %cov, percentage of protein coverage; pep#, number of peptides; PMF, peptide mass fingerprint; Relative fold change, fold change relative to the mock control (calculated by DECODON DELTA 2D software) ±SE. Full information on the phosphoproteins including their classification, peptide sequences, and peak list is given in Supplementary Tables S2 and S4 at *JXB* online.

Table 3. *Regulation of the early cytokinin response phosphoproteins by t-Z in the cytokinin receptor double mutants* ahk2cre1, ahk3cre1, *and* ahk2ahk3

Spot/protein no.	AGI code	Protein name	Relative fold change			
			ahk2ahk3	***ahk2cre1***	***ahk3cre1***	**Wild type**
Significant response apparently mediated by a single cytokinin receptor						
P1	At1g22530	Patellin-2 (PATL-2)	–1.1±0.13	1.5±0.25	1.1±0.04	1.9±0.06
P2	At4g24190	Endoplasmin homologue (SHD)	1.2±0.30	1.4±0.10	1.0±0.30	1.7±0.20
P3	At5g56030	Heat shock protein 81-2	1.5±0.08	1.0±0.05	1.3±0.32	2.0±0.15
P4	At5g11170	DEAD-box ATP-dependent RNA helicase 15	1.4±0.06	1.0±0.03	1.0±0.18	1.5±0.15
P7	X	X	1.0±0.26	1.5±0.07	1.0±0.26	2.5±0.13
P9	At1g09640	Probable elongation factor 1-γ 1	1.5±0.05	1.0±0.12	1.0±0.15	1.5±0.05
P11	At5g60640	Protein disulphide isomerase-like protein	1.0±0.28	–1.4±0.10	–1.1±0.15	–1.8±0.09
P12	AtCg00120	ATP synthase subunit α, chloroplastic	–1.5±0.19	–1.3±0.09	–1.2±0.11	–1.5±0.06
P14	At2g39990	eIF2 (eukaryotic translation initiation factor)	1.0±0.25	–1.1±0.17	–1.5±0.30	–2.0±0.12
P18	At5g56030	Heat shock protein 81-2/3/4	1.2±0.35	–1.4±0.01	–1.0±0.15	–1.5±0.05
P21	At3g51880	HMGB1	–1.5±0.15	–1.3±0.01	1.0±0.20	–1.6±0.14
P27	At1g26630	eIF5A-2 (eukaryotic translation initiation factor)	1.5±0.08	–1.1±0.14	–1.2±0.29	1.5±0.05
P28	At1g20010	Tubulin *β*-5 chain	1.7±0.23	1.0±0.26	1.0±0.05	1.6±0.07
P29	X	X	1.0±0.08	1.6±0.24	1.2±0.11	1.7±0.05
P31	At3g09200	60S Acidic ribosomal protein P0-2	–1.1±0.11	–1.4±0.02	–1.2±0.16	–1.6±0.10
Significant response apparently mediated by two cytokinin receptors						
P13	AtCg00490	Rubisco large chain	1.6±0.21	–1.6±0.57	+/–	–2.0±0.23
	At1g67090	Rubisco small chain 1A, chloroplastic				
P15	At5g14740	β-Carbonic anhydrase 2	1.0±0.18	–1.4±0.14	–1.5±0.34	–1.4±0.10
P19	At3g16420	PBP1	–1.2±0.16	–1.5±0.21	–1.4±0.13	–1.3±0.20
P24	AtCg00490	Rubisco large subunit	+/–	–1.5±0.26	2.0±0.14	1.3±0.15
P25	AtCg00490	Rubisco large subunit	1.4±0.12	1.4±0.21	1.0±0.17	1.3±0.15
P26	At1g76180	Dehydrin ERD14	1.5±0.15	–1.4±0.32	–1.3±0.27	–1.3±0.16
P30	At5g44340	Tubulin β-4 chain	1.4±0.20	1.4±0.07	1.3±0.11	1.4±0.15
Non-significant response to *t*-Z in wild type and/or mutants						
P10	At1g76180	Dehydrin ERD14	+/–	–1.3±0.33	+/–	1.3±0.07
P16	At5g43830	GATase like protein	1.1±0.20	1.0±0.20	–1.1±0.22	–1.5±0.10
P17	At5g56030	Heat shock protein 81-2	1.0±0.16	1.0±0.18	1.0±0.05	1.0±0.30
P20	At5g42790	Proteasome subunit a type-1-A	1.2±0.08	1.0±0.01	1.2±0.14	–1.5±0.08
P22	At3g09200	60S Acidic ribosomal protein P0-2	1.0±0.16	1.0±0.30	1.0±0.19	–1.2±0.20
P23	At1g20440	Dehydrin COR47	1.0±0.02	–1.2±0.34	1.0±0.22	1.1±0.18
	At4g26110	NAP1				

Spot no., spot number (as given in Fig. 1C); AGI code, accession number in the TAIR database; Protein name, entry name according to the NCBI database; Relative fold change, fold change relative to the mock control (calculated by DECODON DELTA 2D software) ±SE; +/–, inconsistent regulation in three biological replicas (down-, up-, and non-regulated in the individual biological replicas). Full information on the phosphoproteins including their classification, peptide sequences, and peak list is given in Supplementary Tables S2 and S4 at *JXB* online.

and *ahk2cre1*), and P24 (*ahk2cre1* and *ahk3cre1*), suggesting receptor interactions in response regulation. Further, loss of consistent regulation in the double mutants for two spots regulated by *t*-Z in the wild type (P16, P20) was observed, implying that simultaneous activity of at least two receptors may be needed for correct regulation of the corresponding proteins. In addition, responses of a fraction of the 15 spots primarily regulated by a single receptor to cytokinin treatment were lower in all three double mutants than in wild-type plants, suggesting they may require simultaneous activity of one or more other receptor(s) for a full response. The highest numbers of regulated spots were found in the *ahk2cre1* (14) mutant, followed by *ahk2ahk3* (11) and *ahk3cre1* (four).

Calcium signalling in regulation of early cytokinin response phosphoproteins

Recognition of ERD14 (P10, P26) and COR47 (P23), in which phosphorylation status and Ca^{2+} binding are reportedly interlinked, as early cytokinin response phosphoproteins suggested a molecular link between cytokinin action and calcium signalling. To test the involvement of calcium signalling in early phosphoproteome regulation by cytokinin, 7-day-old *Arabidopsis* seedlings were treated with 5 μM *t*-Z in the presence and absence of a calcium channel blocker (30 μM D600) and a competitive inhibitor of calcium uptake (60 μM $LaCl_3$) for 15 min, and phosphoproteome alterations were analysed as outlined above. This

resulted in identification of five phosphoproteins in which regulation by *t*-Z was lost in the presence of the calcium signalling inhibitors (Fig. 2), while the remaining pattern of phosphoprotein regulation remained unaltered compared with data given in Table 2.

Comparison of proteome and phosphoproteome data sets

The sets of differentially expressed proteins and phosphoproteins (Fig. 1) included seven overlapping proteins: At3g09200 (T34, P22, P31), At3g16420 (T38, P19), At4g24190 (T2, P2), At5g14740 (T42, P15), At5g60640 (T11, P11), AtCg00120 (T12, T16, P12), and AtCg00490 (T13, T18, P13). However, the apparent pI and molecular mass values were indistinguishable in proteome and phosphoproteome maps for only one of them, endoplasmin homologue (At4g24190), indicating that the proteins displayed in the protein and phosphoprotein maps may be subjected to distinct PTMs affecting their apparent pI, molecular mass, or both.

Classification of identified proteins and phosphoproteins

Identified proteins were categorized using criteria described by Bevan *et al.* (1998). As shown in Fig. 3A, significant fractions of the phosphoproteins are involved in responses to environmental (stress, light, and temperature) stimuli (36%), and signalling and development pathways (27%). Other highly represented functional categories are protein biosynthesis (17%) and transcription regulators (10%). Given previously reported data on the functional significance of phosphorylation of the phosphoproteins identified here, the present data set also indicates that phosphorylation regulation by cytokinins might mainly regulate protein–protein and protein–ligand/substrate interactions (Fig. 3B).

To gain deeper insights into biological processes in which the differentially regulated proteins are involved, the gene ontology (GO) of proteins showing significant ($P < 0.05$), ⩾1.4-fold changes in expression between cytokinin-challenged and control samples was analysed. The results were visualized using BiNGO, a graphical tool enabling GO classes in clustered data to be highlighted (http://www.psb.ugent.be/cbd/papers/BiNGO/) (Fig. 4). The GO

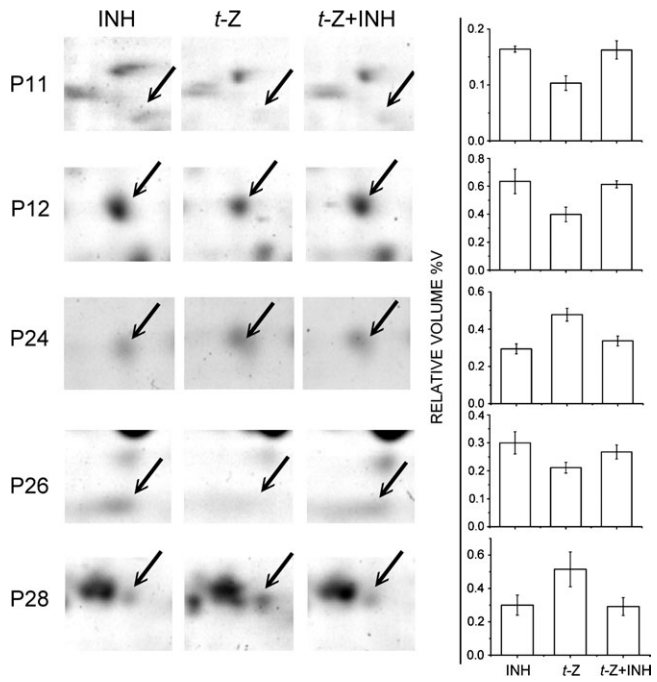

Fig. 2. Effect of calcium signalling inhibitors on regulation by cytokinin of early cytokinin response phosphoproteins. (A) Selected regions of 2D gels showing early cytokinin response phosphoproteins (indicated by arrows) whose regulation by 15 min treatment with 5 μM *t*-Z in *Arabidopsis* seedlings (*t*-Z) was abolished when calcium signalling inhibitors 30 μM D600 and 60 μM $LaCl_3$ were administered simultaneously with 5 μM *t*-Z (*t*-Z + INH). Control samples were treated with the inhibitors only (INH). For details see Materials and methods. Spot numbers as in Fig. 1C and Table 2. (B) Relative volumes of the individual spots.

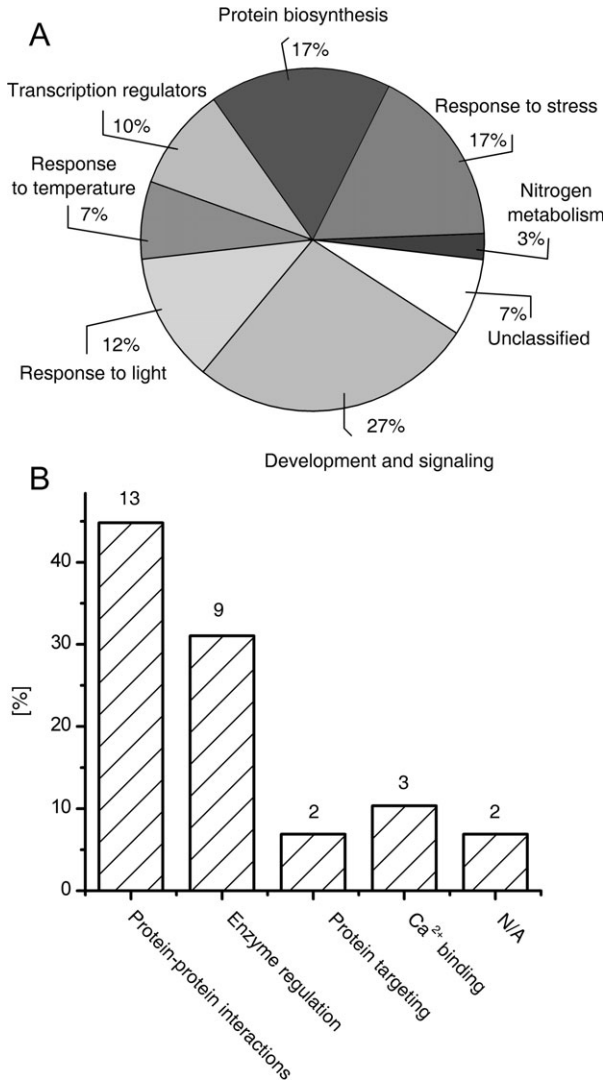

Fig. 3. (A) Classification of the early cytokinin response phosphoproteins according to their cellular functions (Bevan *et al.*, 1998) and (B) molecular processes reportedly controlled by their phosphorylation as deduced from database entries and literature review.

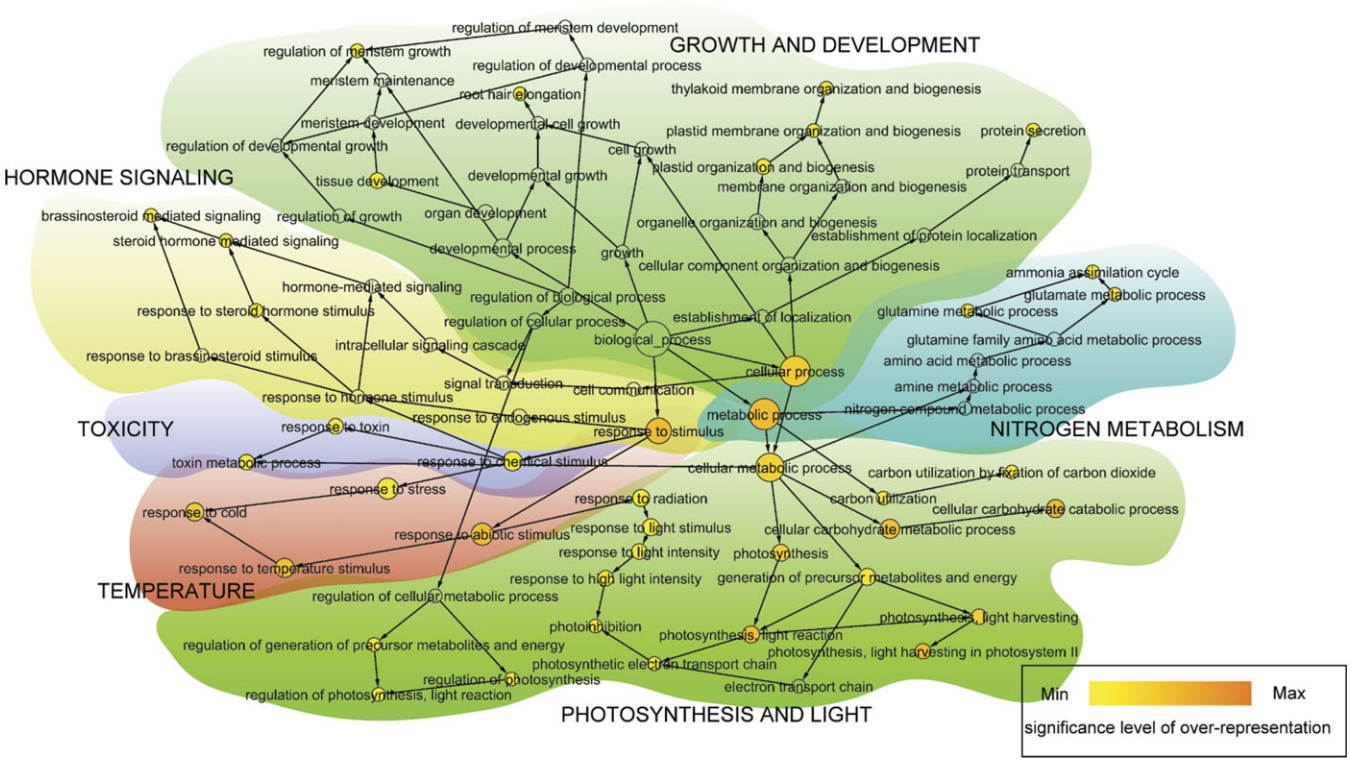

Fig. 4. Gene ontology (GO) analysis of the early cytokinin response proteins in *Arabidopsis* (performed in Cytoscape using BiNGO plugin version 2.3). GO categories that were significantly over-represented among the differentially expressed proteins were identified. The yellow to orange colour of the circles indicates the level of significance of over-represented categories (P=0.05, yellow; P=10^{-7}, orange). The size of the circles is proportional to the number of proteins in each category. Links with low significance were removed manually to reduce complexity of the image.

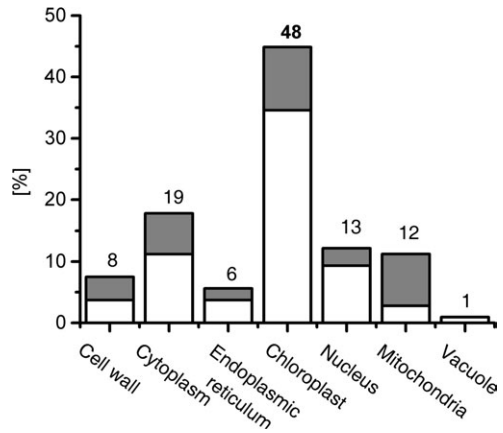

Fig. 5. Subcellular distribution of the early cytokinin response proteins (white) and phosphoproteins (grey) according to the UniProt database (http://www.uniprot.org). The numbers above the columns represent sums of the identified proteins and phosphoproteins located in the respective cellular compartment.

categories that were identified as being significantly over-represented were 'Growth and development' (including subclasses such as 'Thylakoid development' and 'Protein transport'), 'Nitrogen metabolism', 'Hormone signalling', 'Photosynthesis and light' (e.g. 'Response to light stimulus', 'Response to light intensity', and 'Photosynthesis–light reaction and light harvesting'), and 'Toxicity'. Further, 'Temperature response' was the only down-regulated GO category.

The subcellular location of each identified protein was determined according to the TAIR database (http://www.arabidopsis.org), and the results are summarized in Fig. 5. The largest group of proteins was localized to chloroplasts (52%), followed by the cytoplasm (17%) and mitochondria (14%). Among phosphoproteins, 31, 25, and 19% were localized to chloroplasts, the nucleus, and cytoplasm, respectively.

Discussion

Proteomic and phosphoproteomic effects of cytokinin treatment on *Arabidopsis* seedlings were analysed to identify early cytokinin response proteins and phosphoproteins in order to elucidate molecular mechanisms involved in cytokinin action. The identified proteins and phosphoproteins represent a snapshot of early links in various well known cytokinin-regulated signalling circuits and cellular processes. The results also indicate as yet unrecognized links between temperature, calcium, and cytokinin signalling. Comparative analysis revealed differences in both the potency of the four representative cytokinins to trigger the

responses, and the contributions of specific cytokinin receptors to phosphoproteomic responses to *t*-Z treatment.

Phosphoproteome isolation and analysis

A previously established procedure for isolating mammalian and yeast phosphoproteins by affinity chromatography prior to further analysis by 2-DE and MS (Makrantoni *et al.*, 2005) was optimized and employed in this study. The procedure is specific for proteins phosphorylated at serine or threonine residues. Thus, it is capable of detecting most phosphorylated proteins in eukaryotic cells since their pSer:pThr:pTyr and pHis/pTyr ratios are typically 1800:200:1 and 10–100:1, respectively (Klumpp and Krieglstein, 2002; Laugesen *et al.*, 2004). The procedure is reportedly reliable for plant phosphoproteome analysis (Laugesen *et al.*, 2006; Meimoun *et al.*, 2007), and sample preparation for the 2-DE stage of the protocol has been further improved. All proteins identified on the phosphoproteome map show the predicted phosphorylation sites in NetPhos (Blom *et al.*, 1999). Most are already included in the PhosPhAt database (Heazlewood *et al.*, 2008), and the possibility of PTM by phosphorylation of the others has been previously documented (Table 2). Here, phosphorylation for 60S acidic ribosomal protein P0-2 (P22) and endoplasmin homologue (SHD; P2) was confirmed directly, and it was shown that all but two differentially regulated spots are stained by the phosphoprotein-specific stain Phos-Tag (Supplementary Fig. S3 at *JXB* online). Further, phosphorylation-dependent shifts in apparent (SDS–PAGE) molecular masses of COR47 and ERD14 have been reported (Alsheikh *et al.*, 2005), and increases in apparent molecular masses of these proteins after cytokinin stimulation (P10, P23) were observed.

Based on previous reports, the changes in phosphoprotein levels detected here may reflect phosphorylation events mainly involved in regulation of protein–protein or protein–ligand/substrate interactions. Modulation of enzyme activity and specificity by phosphorylation has been shown for histone deacetylase (Pflum *et al.*, 2001), protein disulphide isomerase (Guthapfel *et al.*, 1996), β-carbonic anhydrase (Church *et al.*, 1980), Rubisco (Aggarwal *et al.*, 1993), RNA helicase (Yang *et al.*, 2005), and ATP synthase (Murtazina *et al.*, 2001). Phosphorylation also reportedly mediates association and assembly of protein complexes of Rubisco (Aggarwal *et al.*, 1993), 60S acidic ribosomal protein P0-2 (Naranda and Ballesta, 1991), endoplasmin (Brunati *et al.*, 2000), tubulins (Blume *et al.*, 2009), proteasome subunits (Umeda *et al.*, 1997), and heat shock proteins (Picard, 2002). Down-regulation of eIF2 (P14), and up-regulation of eIF5A-2 and probable elongation factor 1-γ1 (P28, P9) are consistent with previously reported stimulation of proteosynthesis by cytokinins as eIF2 reportedly inhibits initiation of proteosynthesis in its phosphorylated form (Zhang *et al.*, 2008) while phosphorylation of eIF5 and EF-1 reportedly stimulates formation of initiation complexes (Majumdar *et al.*, 2002) and enhances proteosynthesis (Belle *et al.*, 1995), respectively. Further, phosphorylation reportedly promotes NAP1 import into the nucleus (Calvert *et al.*, 2008), while HMGB1 requires phosphorylation for export from the nucleus (Youn and Shin, 2006). Finally, several phosphoproteins reportedly related to other signalling pathways were identified that may be involved in as yet unrecognized branches of cytokinin signalling and/or as molecular players in cross-talk between cytokinins and other stimuli. For example, PATL-2 (P1) is reportedly involved in membrane trafficking events (Peterman *et al.*, 2004) and its phosphorylation has been localized to its phosphoinositide-binding pocket (Jones *et al.*, 2009). This is consistent with recent indications of a role for intracellular trafficking in cytokinin signalling (Dortay *et al.*, 2008).

Temperature perception

A novel theme highlighted by the present analysis is differential regulation by cytokinins of proteins and phosphoproteins reportedly involved in responses to high and low temperatures (Nylander *et al.*, 2001; Sung *et al.*, 2001; Bae *et al.*, 2003; Goulas *et al.*, 2006; Lim *et al.*, 2006; Sasaki *et al.*, 2007). The proteins include heat shock cognate 70 kDa protein 1, fructose-1,6-bisphosphatase, phosphoribulokinase, phosphoglycerate kinase, 60S acidic ribosomal protein P0-2, putative ribonucleoprotein At2g37220, and cold shock domain protein 2, and the phosphoproteins comprise heat shock protein 81-2, 60S acidic ribosomal protein P0-2, and dehydrins COR47 and ERD14. Interestingly, all the proteins were down-regulated by cytokinins, suggesting there may be shared components of cytokinin and temperature signalling pathways. In plants, mechanisms underlying temperature perception are poorly understood (Penfield, 2008). However, a two-component signalling pathway is known to act in temperature perception in cyanobacteria (e.g. *Synechocystis*), with a histidine kinase perceiving and relaying temperature signals (Suzuki *et al.*, 2000), and a two-component signalling pathway is the main known component of cytokinin signalling chains in plants. Accordingly, a role for cytokinins in responses to cold stress was recently deduced from the apparent attenuation of cytokinin signalling under cold stress (Argueso *et al.*, 2009). Decreases in levels of endogenous cytokinins in heat shock-treated plants have also been documented (Hare *et al.*, 1997), and Burkhanova *et al.* (2001) found that responses to BA were enhanced in heat-shocked *Arabidopsis thaliana*, prompting speculation that heat shock proteins may be involved in cytokinin signalling.

Chloroplast biogenesis and function

The differentially regulated proteins and phosphoproteins detected in the presented experiments include a remarkably high percentage of proteins located to chloroplasts—45%—compared with 7.9% chloroplast proteins predicted for the whole genome (Bevan *et al.*, 1998). They reflect most processes involved in chloroplast biogenesis and function, including: mRNA processing; protein biosynthesis, folding, and degradation; light and dark reactions of

photosynthesis; carbon utilization; carbohydrate metabolism; glycolysis; fatty acid biosynthesis; and stress responses. Both the number and variety of functions in which they are implicated substantially exceed estimates obtained in two previous proteomic analyses of cytokinin action. In *Physcomitrella patens*, four early response phosphoproteins located to chloroplasts were identified following short cytokinin treatment (Heintz *et al.*, 2006), and 10 up-regulated chloroplast proteins were found when effects of continuously increasing endogenous cytokinin levels in dark-grown *Arabidopsis* seedlings were investigated (Lochmanová *et al.*, 2008). For two of the *Physcomitrella* phosphoproteins (Rubisco large subunit and carbonic anhydrase) functional matches are present in our data set. The high number of early cytokinin response proteins and phosphoproteins located in chloroplasts might indicate an as yet uncharacterized direct branch in cytokinin signalling responsible for cytokinin action in chloroplasts. Consistently, 10 interactors of cytokinin receptors have been located to chloroplasts (Dortay *et al.*, 2008), and one of them, Rubisco small chain 1A (At1g67090), was identified here as a constituent of the differentially regulated spot P13. Brenner *et al.* (2005) have proposed that rapid transfer of cytokinin signals to plastids, or direct perception and interpretation of the signals by the plastids, may explain the fast regulation of five chloroplast transcripts by cytokinin treatment. Interestingly, the chloroplast cytokinin pool has been found to be dynamic (Benková *et al.*, 1999), and compartmentation into chloroplasts of some cytokinin biosynthesis and metabolism pathways has been reported (Brzobohatý *et al.*, 1993; Kristoffersen *et al.*, 2000; Takei *et al.*, 2004; Kiran *et al.*, 2006).

Chromatin remodelling and nuclear proteins

Novel insights into possible involvement of cytokinins in chromatin remodelling were obtained by identifying HMGB1, histone deacetylase HDT2, and possibly NAP1;1 as early cytokinin response phosphoproteins. Interestingly, cytokinin response genes have been found to be up-regulated in *hmgb1* knockout mutants (Lildballe *et al.*, 2008), while NAP1;2 protein reportedly interacts with ARR7 (Dortay *et al.*, 2008), and NAP1 proteins are positive regulators in the ABA signalling pathway (Liu *et al.*, 2009). The expression level of genes involved in chromatin remodelling has been found to change after just 120 min cytokinin treatment (Brenner *et al.*, 2005), clearly demonstrating the power of proteomic profiling for identifying primary events in cytokinin action.

Cytokinin and cytokinin receptor specificity in eliciting proteomic/phosphoproteomic alterations

The present experiments showed that the representatives of the four cytokinin classes had largely similar qualitative proteomic and phosphoproteomic effects, although the extent of up- or down-regulation varied for most proteins and phosphoproteins. The varying degree of responsiveness might reflect the molecular basis of differential activities of different cytokinin classes previously observed in various physiological experiments (e.g. Mok *et al.*, 1978; Sujatha and Reddy, 1998; Lexa *et al.*, 2003; Hradilová *et al.*, 2007), and is consistent with distinct specificities of the individual cytokinin receptors for individual cytokinin moieties (Spíchal *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004; Romanov *et al.*, 2006). Generally, the highest degree of differential regulation of proteins and phosphoproteins was elicited by BA and TDZ (reportedly the most potent cytokinin in activation of the cytokinin primary response gene *ARR5* in *Arabidopsis*; Spíchal *et al.*, 2004). The present study is the first global comparison of the effects of all four classes of cytokinins. However, Rashotte *et al.* (2003) compared the effects of BA and *t*-Z on expression profiles in *Arabidopsis* and found that while both cytokinins up-regulated largely overlapping sets of genes, far fewer genes were found to be down-regulated by *t*-Z than by BA, concluding that some of these genes may be specifically down-regulated by BA or not actually regulated by cytokinin.

The profiling of *t*-Z action in the cytokinin receptor double mutants *ahk2ahk3*, *ahk2cre1*, and *ahk3cre1* provides first insights into the specificity of outputs of specific cytokinin receptors at the proteome level. Most of the identified phosphoproteins were found to be differentially regulated primarily by a single cytokinin receptor. However, indications of inter-receptor cooperation were seen for some of the differentially regulated phosphoproteins. Detection of a significant number of phosphoproteins regulated by two individual cytokinin receptors was consistent with reportedly overlapping functions of the cytokinin receptors (Inoue *et al.*, 2001; Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.*, 2006). Defining the output of cytokinin receptors as the number of phosphoproteins they apparently regulate, their output decreases in the order AHK3>AHK4/CRE1/WOL1>>AHK2. This observation is consistent with degrees of involvement of the cytokinin receptors in various biological processes previously found in morphological, physiological, and molecular analysis of cytokinin receptor mutants (Riefler *et al.*, 2006).

Calcium signalling

The present work provides the first molecular link between cytokinin action and signalling pathways involving modulation of free Ca^{2+} levels, showing that early cytokinin response phosphoproteins include ERD14 and COR47, for which correlations between phosphorylation status and Ca^{2+} binding have been demonstrated (Alsheikh *et al.*, 2005). In addition, it is shown that inhibition of calcium signalling abolishes cytokinin regulation of several phosphoproteins, further supporting the interlinking of cytokinin and calcium signalling. *In planta*, inhibition of calcium signalling disrupts cytokinin-induced bud formation in the moss *Funaria* (Saunders and Helper, 1983). Ca^{2+} signalling is reportedly involved in the transduction of diverse abiotic, biotic, and developmental stimuli including

temperature and plant hormones (Sanders *et al.*, 2002). In this context, increases in the phosphorylation of SHD and HSP 81-2 (P2, P3), proteins related to the hsp-90 family, whose members are known to be autophoshorylated in the presence of Ca^{2+} (Csermely and Kahn, 1991), were also observed. Further, Ca^{2+}-mediated signalling may represent a rapid mechanism of transmitting cytokinin signals into chloroplasts. It has long been established that chloroplast-localized physiological processes are subject to regulation by Ca^{2+} and, accordingly, a Ca^{2+}-sensing receptor has been localized to the chloroplast and found to modulate cytoplasmic Ca^{2+} concentrations (Nomura *et al.*, 2008; Weinl *et al.*, 2008).

Conclusion

In conclusion, a novel proteome- and phosphoproteome-wide view of changes in abundance of proteins and phosphoproteins is presented that might be functionally relevant for the many biological processes regulated by cytokinins. Importantly, the results indicate as yet unrecognized links between temperature, calcium, and cytokinin signalling. Interlinking of cytokinin and calcium signalling is further supported by loss of cytokinin regulation of several phosphoproteins following inhibition of calcium signalling. Rapid regulation of a number of chloroplast phosphoproteins suggests a currently uncharacterized direct signalling chain responsible for cytokinin action in chloroplast. Comparative analysis of the representatives of the four cytokinin classes revealed largely similar regulation patterns in the 7-day-old *Arabidopsis* seedlings. First insights into the specificity of cytokinin receptors on phosphoproteomic effects were obtained from analysis of cytokinin action in the set of cytokinin receptor double mutants. The presented data provide a new framework for further detailed investigations, using, for example, mutants and transgenic plants, of molecular mechanisms involved in cytokinin action. Further identification of kinase(s) and phosphatase(s) involved in phosphorylation and dephosphorylation events triggered by cytokinins and elucidation of their relationships to cytokinin receptors are important challenges for future work. Interestingly, two protein kinases have been identified as AHK2 and AHK4 interactors, and a phosphatase as an ARR4 interactor (Dortay *et al.*, 2008).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Schematic representation of the experimental design.

Figure S2. Confirmation of peptide phosphorylation.

Figure S3. Detection of phosphoproteins isolated using the PhosphoProtein Purification Kit and separated by 2-DE.

Table S1. Early cytokinin response proteins of *Arabidopsis* (an extended version of Table 1)

Table S2. Early cytokinin response phosphoproteins of *Arabidopsis* (an extended version of Table 2)

Table S3. Detailed MS information on the early cytokinin response proteins of *Arabidopsis*.

Table S4. Detailed MS information on the early cytokinin response phosphoproteins of *Arabidopsis*.

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